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EXH. 6

The bioactivation of CB 1954 and its use as a prodrug in antibody-directed enzyme prodrug therapy (ADEPT)

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Abstract

Walker cells *in vivo* or *in vitro* are exceptionally sensitive to the monofunctional alkylating agent CB 1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide). The basis of the sensitivity is that CB 1954 forms DNA interstrand crosslinks in Walker cells but not in insensitive cells. Crosslink formation is due to the aerobic reduction of CB 1954 to form 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide by the enzyme DT diaphorase. The 4-hydroxylamine can not crosslink DNA directly but requires further activation by a non-enzymatic reaction with a thioester (such as acetyl coenzyme A). As predicted from their measured DT diaphorase activities, a number of rat hepatoma and hepatocyte cell lines are also sensitive to CB 1954. However, no CB 1954-sensitive tumours or cell lines of human origin have been found. This is because the rate of reduction of CB 1954 by the human form of DT diaphorase is much lower than that of the Walker enzyme (ratio of $k_{cat} = 6.4$). To overcome this intrinsic resistance of human cells towards CB 1954 a number of strategies have been developed. First, analogues have been developed that are more rapidly reduced by the human form of CB 1954. Second, the cytotoxicity of CB 1954 can be potentiated by reduced pyridinium compounds. Third, a CB 1954 activating enzyme can be targeted to human tumours by conjugating it to an antibody (ADEPT). A nitroreductase enzyme has been isolated from *E. coli* that can bioactivate CB 1954 much more rapidly than Walker DT diaphorase and is very suitable for ADEPT. Thus CB 1954 may have a role in the therapy of human tumours.

1. Introduction

CB 1954 (5-(aziridin-1-yl)-2.4-dinitrobenzamide) was, until recently, an enigma. This agent represents one of the few examples of a compound showing a real anti-tumour selectivity. Whilst chemically only a monofunctional alkylating agent (by virtue of its single aziridine function), CB 1954 exhibited a dramatic and highly selective activity against the rat Walker 256 tumour and could actually cure this tumour. Such selectivity was unexpected from a monofunctional alkylating agent and it was evident that the sensitivity of the Walker tumour towards CB 1954 pointed to a unique biochemical feature. The prospect that a human tumour could be found that shared the Walker tumour's sensitivity has made the mechanism of action of CB 1954 the subject of continual interest for over 20 years. CB 1954 has been described as 'a drug in search of a human tumour to treat' [1].

2. Historical background

A large series of N-substituted ethyleneimine derivatives (aziridines) were synthesized in the early 1950's and investigated for their tumour growth-inhibitory activity against the Walker 256 carcinoma

in vivo [2, 3]. The most pronounced anti-tumour effects were observed with molecules containing at least two ethyleneimine residues. An exception to this requirement for difunctionality was the monoethyleneimine derivative, 1-(1-aziridinyl)-2,4-dinitrobenzamide or CB 1837, which was found to have significant anti-tumour activity. CB 1837 was found to have a therapeutic index (TI = 10) that was comparable to many agents in current clinical use, for example, chlorambucil (TI = 7), cyclophosphamide (TI = 22), melphalan (TI = 9) and cisplatin (TI = 14).

During the examination of a large series of structurally related compounds, a derivative carrying a carboxamide substituent (CB 1954) was shown to have an even higher therapeutic index against the Walker 256 carcinoma (TI = 70). This was higher than any compound known [4]. CB 1954 had arisen in an attempt to make CB 1837 more water soluble by the introduction of the carboxamide group but slight alterations in the structure of CB 1954 often led to complete loss of activity. Whilst CB 1954 exhibited high potency and selectivity against this tumour model, it lacked any significant effect on a large number of other transplantable tumours [5, 6].

2.A *The anti-tumour activity and toxicity of CB 1954*

Although CB 1954 was shown to have high potency and specificity of action against Walker tumour cells both *in vivo* and *in vitro*, it was established from an early stage that CB 1954 was ineffective in a range of animal tumours that respond to the growth inhibitory effects of difunctional alkylating agents [5–7]. That this sensitivity was also absent in human cells was confirmed [8, 9], where continuous exposure to CB 1954 was found to be more growth inhibitory than cytotoxic. Workman *et al.* [1] reconfirmed that CB 1954 had essentially little activity against a range of rodent and human tumours *in vivo*. Administration of the maximum tolerated dose produced essentially no anti-tumour activity in a range of xenografts *in vivo*.

In addition to its potent and specific activity, it exhibited minimal toxic effects on the haematopoietic system. Pathological effects, at toxic doses in rodents, were observed in the liver and urinary tract epithelium [10]. This is in contrast to the intestinal epithelium toxicity observed with many alkylating nitrogen mustards [5]. CB 1954 was found to be almost 10 times less toxic in the mouse than in the rat. This was reflected in the higher therapeutic index of this compound against the Walker tumour when grown and treated in mice [11].

CB 1954 was entered into clinical trials in 1970 owing to its high therapeutic index when tested against the Walker tumour *in vivo*, despite its limited effectiveness against some other experimental tumours. No regression was observed in any of the cases. The most severe side effect to treatment was diarrhoea, with no bone marrow toxicity or liver dysfunction being observed (E. Wiltshaw, unpublished clinical results). These side effects had been similarly observed in animal tests [10].

2.B *Metabolism studies with CB 1954* in vivo

To understand the selective toxicity of CB 1954 against the Walker 256 carcinoma, metabolism studies were carried out in the hope of identifying metabolites that might be implicated in its anti-tumour activity.

Studies with CB 1954 in the rat concluded that unchanged CB 1954 was the most abundant radioactive constituent in the urine of animals treated with tritium-labeled drug [12]. The principle urinary metabolite was the 4-amine product, 5-(aziridin-1-yl)-4-amino-2-nitrobenzamide. This was also tumour inhibitory, but much less so than CB 1954 (TI = 16). Other urinary metabolites identified were 5-amino-2,4-dinitrobenzamide and 5(2′-hydroxyethyl)amino-2,4-dinitrobenzamide, which had been previously identified [13]. Non-polar metabolites accounted for at least 85% of the urinary radioactivity from CB 1954-treated rats in this study. Minor quantities of polar metabolites were recovered that were not identified.

The isomer of the 4-amine product, the 2-amine, had previously been suggested to be the metabolite of CB 1954 responsible for its activity [14]. The structural resemblance that existed between this amine and *in vivo* antagonists of the antitumour ac-

tivity of CB 1954, such as the purine precursors (AICA) [13], suggested this candidate to be the more likely metabolite. However, the 2-amine product was not detected in the urine and exhibited only minimal inhibitory action on the growth of the Walker tumour *in vivo*.

More recently, the pharmacokinetics and metabolism of CB 1954 have been studied in mice [1, 15]. The presence of all previously identified metabolites was confirmed. It was proposed that reduction of the nitro groups by 1-electron reduction reactions to form reactive intermediates might be mediated intracellularly and account for the cytotoxicity of CB 1954. This was investigated by the use of allopurinol, which is an inhibitor of the enzyme xanthine oxidase, which functions as a nitroreductase (by 1-electron transfer) and is thus implicated in the bioactivation of CB 1954. The *in vivo* antagonist, 2-phenyl-AICA, was shown not to alter blood concentrations of CB 1954 or its metabolites. Earlier studies by 13, had concluded that no chemical interaction between neutralized AICA hydrochloride and CB 1954 occurred *in vitro*.

In summary, metabolism studies were inconclusive. No single product was identified that could account for the selectivity or cytotoxicity of CB 1954.

2.C Investigations into the selective activity of CB 1954 against the Walker 256 carcinoma

Preliminary studies on the mechanism of action of CB 1954 suggested that the sensitivity observed towards this compound was typical of a difunctional alkylating agent [6, 16, 17]. It was proposed that the monofunctional CB 1954 might be converted by a nitroreductase activity present in the Walker tumour to a compound capable of reacting difunctionally with DNA [6]. Inter or intrastrand cross-link formation is generally accepted as the most toxic lesion that will inhibit DNA function, eventually resulting in cell death. However, because the formation of a difunctional alkylating agent could not be verified, other lines of investigation were pursued. Studies on the mechanism of action of this compound were aimed at identifying the biochemical feature of the Walker tumour that made it so sensitive to this agent. Human tumours could then be chosen on a rational basis according to this specific property.

Preliminary experiments explored the possible role of CB 1954 as an anti-metabolite, as the dose response curve was similar to that obtained with anti-metabolites such as methotrexate [6]. In addition, the selective antitumour effects of CB 1954 against the growth of the Walker 256 carcinoma both *in vivo* and *in vitro* were shown to be reversed by a variety of aminoimidazolecarboxamides, anthranilamide, adenine and 2,4-dichlorophenol [6, 13, 18]. The protection by 5(4)-aminoimidazole-4(5)-carboxamide (AICA) led to the view that CB 1954 acted as a purine antimetabolite [13] as a structural similarity existed between the proposed structure that would result from ring closure between the reduced nitro group (to the amine) and the aziridine ring, and AICA. The phosphoribosyl derivative of AICA is a purine precursor in the *de novo* purine biosynthetic pathway and it was suggested that CB 1954 might be competing for the same receptor site as this compound. It was proposed that it might be interfering with an early stage in purine biosynthesis. However, direct measurement of the inhibition of purine biosynthesis by CB 1954 could not be shown. However, these protectors were shown to reverse the inhibition of thymidine incorporation into DNA by this agent and protect against CB 1954 toxicity *in vivo* [13, 17]. It was proposed that this alleviation by AICA was by the provision of products for the reaction blocked by CB 1954 [17]. The 2-phenyl derivative of AICA (2-phenyl-5(4)-aminoimidazole-4(5)-carboxamide) (2-phenyl-AICA), was shown to be the most potent protector, reducing the therapeutic index of CB 1954 by a factor of 30 [18]. Other protectors were generally unrelated both functionally and structurally. No effects on cellular uptake or energetics (AICA) were shown in addition to lack of post protection of CB 1954 toxicity [18]. These compounds, however, did not protect against the difunctional compound, melphalan [19].

CB 1954, at high doses, was subsequently shown to inhibit the enzyme ribonucleotide reductase [20], an enzyme critically involved in the synthesis of deoxyribonucleotides. It was proposed that protection by 2-phenyl-AICA and other compounds was

by competition with CB 1954 for binding to this enzyme. However, none of these findings explained why CB 1954 was selectively toxic to the Walker 256 carcinoma but only proposed a possible mechanism of action of this compound.

In a further attempt to unravel the mechanism of action of CB 1954, a series of investigations were carried out to determine its effects on cAMP levels in treated cells. A diverse set of cellular responses are mediated by cAMP, an increase in which may ultimately lead to for example, growth inhibition. Difunctional alkylating agents, and not anti-metabolites, were shown to elevate cAMP levels in sensitive tumour cells and this was shown to be proportional to the dose required to produce growth inhibition [21, 22]. This effect was not observed in resistant cells and monofunctional alkylating agents could not mediate this elevation. The exception was CB 1954. Inhibition of a phosphodiesterase enzyme in the degradation and hydrolysis of cAMP was shown to occur where growth inhibition and elevated cAMP levels were observed in sensitive (in which this enzyme was better represented) but not resistant cells treated with chlorambucil. However, CB 1954 was found not to inhibit phosphodiesterases, neither did it affect the activity of the enzyme involved in cAMP synthesis, adenylate cyclase [23]. It was proposed that Walker cells could regulate their cAMP levels by leakage to the exterior. If this mechanism could be inhibited by CB 1954 it could account for the specificity of this agent towards these cells.

In a further study, the effects of alkylating agents on cAMP-dependent protein kinase activity was determined in Walker cells [24]. Chlorambucil was found to activate the enzyme by the release of the catalytic subunit of the protein kinase at concentrations that caused complete inhibition of cell growth. A similar effect was produced by CB 1954 and this could be reversed by the tumour growth inhibitory antagonist, 2-phenyl-AICA. Walker cells that had been made resistant to CB 1954 contained predominantly one type of protein kinase that had a lower dependency for cAMP. This explained the decreased stimulation of protein kinase by cAMP in this cell line [25] and presumably, the decreased cytotoxicity of CB 1954.

CB 1954 was shown to potentiate the cytotoxic effects of melphalan, without the preincubation in hypoxia normally required by nitroaromatic radiosensitizers [26]. It was suggested that inhibition of a DNA repair process may be involved and it was proposed that CB 1954 may be acting as an inhibitor of poly(ADP-ribosylation) as it had structural similarities to known inhibitors of this enzyme that is thought to be involved in the repair of DNA. However, no inhibition in the depletion of NAD^+ levels following exposure to melphalan was effected by CB 1954 (the transfer of ADP-ribose from NAD^+ to chromosomal proteins catalyzed by this enzyme would be required for repair synthesis of damaged DNA).

2.D The hypoxic cell cytotoxicity of CB 1954

A characteristic of many aromatic compounds containing a conjugated nitro group is their ability to sensitize hypoxic cells and CB 1954 possessed this characteristic [27]. The ability to sensitize hypoxic mammalian cells has been shown to be mainly influenced by the one-electron reduction potential of the compound, i.e., electron affinity. CB 1954 had a similar one electron reduction potential to misonidazole, but its ability to sensitize hypoxic cells was greater – as indicated by the lower level required for a given enhancement ratio [27]. This difference was abolished by the presence of the tumour growth inhibitory antagonist, 2-phenyl-AICA. It was suggested that the additional sensitization by CB 1954 was due to other cytotoxic properties associated with the compound as CB 1954 did not alter the radiosensitization effected by misonidazole. The order of effectiveness as chemotherapeutic agents of some CB 1954 analogues was the same as their efficiency as hypoxic cell radiosensitizers, but was not related to their one electron reduction potentials. It was shown previously that the reactivities of the aziridine ring were similar in all three compounds. Although partition coefficients differed, a previous study had shown this not to be a determinant of hypoxic sensitization in mammalian cells *in vitro*. In a further study, the relative sensitizing efficiencies of a series of analogues of CB 1954 in which the indi-

vidual contribution of the aziridinyl group and other ring substituents to the sensitizing activity were assessed [28]. In contrast to CB 1954, the sensitization by a mono-nitro analogue was shown to be potentiated in the presence of 2-phenyl-AICA. Multiple mechanisms of radiosensitization were thought to contribute to the unusually high efficiency of this property of CB 1954.

2.E Structural requirements of CB 1954

The parent compound, 1-aziridinyl-2,4-nitrobenzene (CB 1837), was modified with various substituents in relation to the aziridine group (on the C1 position of this compound) [4]. Electron withdrawing nitro groups were originally added to increase the chemical reactivity of the aziridino group, since the attraction of the lone pair of electrons from the nitrogen atom would increase the ring strain. However, the ortho-nitro group could not be replaced by another electron-withdrawing group or a heterocyclic nitrogen atom, without loss of activity. Replacement of the para-nitro group with a cyano group resulted in the retention of tumour growth-inhibitory effectiveness, with, however, a decrease in chemotherapeutic index due to increased toxicity. Replacement of this group with a sulphonamido group also resulted in loss of activity. The addition of a second aziridine group at the C5 position, rendering the compound difunctional, resulted in a loss of carcinostatic activity. A cyano, carbomethoxy and carboethoxy group in this position resulted in reduced or marginal activity. The only modification of the parent structure (CB 1837) which gave a compound with enhanced potency and an increased chemotherapeutic index, was the insertion of a carboxamide group at this C5 position to produce CB 1954.

An explanation for the potency and selectivity of this compound was that the amide might undergo selective hydrolysis within cells of the Walker tumour. This would give the free acid that would be completely ionized at intracellular pH and unlikely to diffuse out again. N-substituted (5-carboxylic) ester derivatives were prepared with special regard to influencing solubility, distribution characteristics and stability of the amide towards hydrolysis [14]. None of the substituted amides were found to have activity that approached the benzamide derivative. In general, substitution that would be expected to increase lipid solubility decreased toxicity. Variations in the carboxamide group did not affect the chemical reactivity of the alkylating group, leading to the conclusion that variations in biological activity were not due to differences in this reactivity. There was found to be no correlation between the toxicities of the compounds or their rates of chemical reaction with a nucleophile and their anti-tumour activities.

Electron withdrawing groups were introduced to examine the effects of decreased hydrolytic stability on anti-tumour activity. Only the N-bromoethyl derivative at the C5 position was shown to have a significantly increased therapeutic index (TI = 46). An increase in hydrolytic stability did not improve anti-tumour activity significantly. None of the synthesized analogues showed significant growth-inhibitory activity when tested against the ADJ/PC6A mouse plasmacytoma, which is highly sensitive towards difunctional alkylating agents.

In an extension of the earlier analogue work, it was proposed that replacement of the alkylating function with other suitable cytotoxic groups might facilitate the localization of activity within the cell [29]. However, replacement of the ethyleneimine alkylating function of CB 1954 with another alkylating function or addition of a second alkylating centre resulted in compounds that were less effective than CB 1954 against the Walker carcinoma *in vivo*. It was proposed that a possible route of metabolism might be the reduction of the nitro groups to amino derivatives, possibly by way of hydroxylamino intermediates. These compounds could possess latent activity and might be more readily produced in tumours containing higher levels of nitro-reductase activity than the surrounding normal tissue [29].

In summary, CB 1954 has a high structural specificity. It was established from an early stage that the 4-nitro group was essential for the activity of CB 1954 and it could not be replaced by another electron-attracting group. There was no obvious structure-activity relationship.

3. The mechanism of action of CB 1954

The specificity of CB 1954 against Walker cells has also been demonstrated in tissue culture; thus eliminating any role the host may play in activating the drug. *In vitro* Walker cells are about a 100,000 fold more sensitive towards CB 1954 than are, for example, Chinese hamster V79 cells (Fig. 1). It was in this cell system that the actual reason why Walker cells were sensitive to this drug was first shown. It was demonstrated that CB 1954 formed DNA-DNA interstrand crosslinks in Walker cells but not in the insensitive V79 cells [30] (Fig. 2). Thus, in Walker cells, CB 1954 is being converted from a monofunctional agent to a difunctional agent. Co-culturing Chinese hamster V79 cells with Walker cells in the presence of CB 1954 resulted in the sensitization of the V79 cells towards CB 1954. Crosslinks were now present in their DNA [31], indicative of the formation in Walker cells, of a diffusible toxic metabolite of CB 1954. It was suggested that this activation was occurring by reduction of the nitro groups of CB 1954 to provide an additional reactive centre [30] and it was subsequently reported that CB 1954 was less toxic and mutagenic in a nitro-reductase-deficient strain of *E. coli* [32]. That this was, indeed, the case was demonstrated when the nitroreductase enzyme responsible for this action was purified from Walker cells and the activated form of CB 1954 identified.

3.A The bioactivation of CB 1954

The purification of the activating enzyme from Walker cells and the identification of this nitroreductase as DT diaphorase is described below. This enzyme, in the presence of NADH or NADPH, catalyses the aerobic reduction of CB 1954 to its 4-hydroxylamino derivative, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide [31] (Fig. 3). These initial studies also indicated some reduction of CB 1954 to the 4-amino derivative. However this compound was shown not to account for the observed cytotoxicity [31] and was not seen in later studies with more highly purified DT diaphorase [33, 34]. 5-(Aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide is highly cytotoxic, even to those cells resistant to CB 1954 and can form interstrand crosslinks in their DNA. It is the formation of this compound that accounts for the sensitivity of Walker cells towards CB 1954. Irrespective of the ability to bioactivate CB 1954, all the cell types so far examined have a comparable sensitivity towards the reduced 4-hydroxylamino derivative [33].

While 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide can produce DNA-DNA interstrand crosslinks in cells it cannot form these lesions in naked DNA [31]. It was suggested that in cells there is a further activation step that converts 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide to the proximal, DNA crosslinking, cytotoxic species. This notion was supported by the absence of a linear dose response in crosslinking in cells treated with this compound consistent with the saturation of this second activation step [31]. An enzymatic esterification and activation of the hydroxylamine, analogous to that formed by metabolism of 4-nitroquinoline-N-oxide and N-acetylaminofluorene, was proposed [31]. In fact, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide can be activated non-enzymatically, to a form capable of reacting with naked DNA to produce interstrand crosslinks, by a direct chemical reaction with acetyl-coenzyme A and other thioesters [35] (Fig. 3). The ultimate, DNA reactive, derivative of CB 1954 is probably 4-(N-acetoxy)-5-(aziridin-1-yl)-2-nitrobenzamide [35] (Fig. 3). Another product of this reaction with thioesters is the 4-amino derivative. Formation of this product is actually in competition with the activation reaction [35] (Fig. 3). Interestingly, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide is the major urinary metabolite of CB 1954 in the rat [12].

The bioactivation of CB 1954 results in a vast increase in its cytotoxicity and the resulting dose modification can be up to 100,000 fold. This is greater than would be predicted by a conversion of a mono- to a difunctional agent. Where monofunctional congeners of difunctional agents are available, as with half mustards and monofunctional platinum compounds, the dose modification for equitoxicity is seen to be only around 50–200 fold [36, 37]. Observations regarding the formation of DNA interstrand crosslinks and their properties may ex-

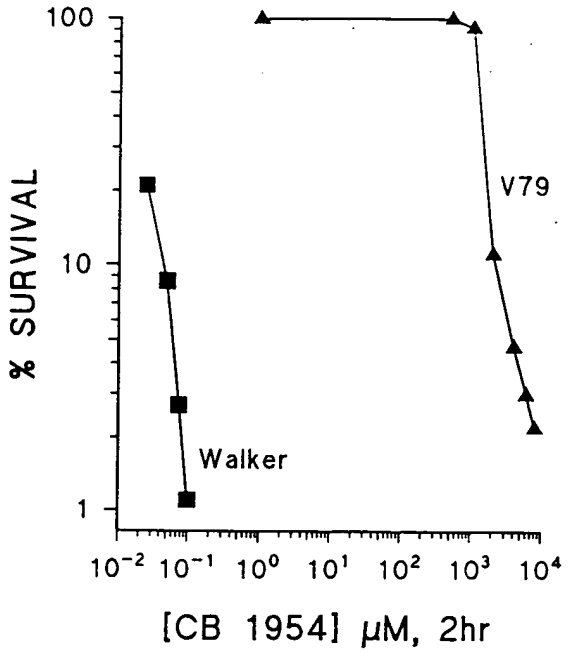

Fig. 1. The effect of CB 1954 on the colony forming ability of Walker 256 cells or Chinese hamster V79 cells. All treatments were for 2 hr at 37° C. The concentration of CB 1954 is plotted on a logarithmic scale. Data from [30].

plain why cells able to bioactivate CB 1954 are so cytotoxically affected.

First, Walker cells have an inherent sensitivity to any agent that can produce DNA interstrand crosslinks [36]. However, Walker cells that have lost this sensitivity to crosslinks are still very sensitive to CB 1954 and are still about 10,000 fold more sensitive than V79 cells [36].

Second, the CB 1954 induced interstrand crosslink is formed with a very high frequency and can contribute up to 70% of the total lesions [38]. This frequency is much higher than that reported for other agents. For example, interstrand crosslinks represent 2% or less of the total DNA reactions of Cisplatin or Carboplatin. The interstrand crosslink is, in terms of molar efficacy, a more intrinsically toxic lesion than are single-strand diadducts and monofunctional lesions [39]. An agent that produced a very high proportion of crosslinks would be expected to be more toxic than one that produced only a low frequency. The crosslink frequency did vary with the treatment protocol used [38]. This is probably due to saturation of the bioactivation stages at high doses of CB 1954 and it appears that it is the second step that is rate limiting in those cells capable of generating the hydroxylamine [31].

Third, the crosslinks are poorly repaired which may be cause them to be even more intrinsically cytotoxic than those induced by other difunctional agents [38].

Fourth, as a consequence of the bioactivation of the CB 1954, there is a 10-fold increase in the amount of DNA bound drug in Walker cells, as compared to cells which can not reduce CB 1954 [38].

The unusual properties of the CB 1954-induced interstrand crosslink suggests that this lesion is unlike those formed by other agents. The interstrand crosslink lesion(s) induced by CB 1954 have yet to be fully identified. However, 4-hydroxylamine (after activation as detailed above) reacts predominantly with the C8 position of deoxyguanosine. In DNA this would leave the aziridine function poised to react on the opposing strand and form the observed crosslinks. Molecular modelling studies indicate that this second arm reaction will preferentially be on the O6 position of a deoxyguanosine on the opposite strand of DNA (Fig. 4). Such a C8-O6 DNA interstrand crosslink would be unique and is not produced by other types of alkylating or platinating agents and may account for its unique properties.

These properties coupled with the selectivity of the bioactivation step by DT diaphorase, explains why CB 1954 was so exceptionally effective as an anti-tumour agent in the rat and capable of curing the Walker carcinoma.

3.B The nitroreductase enzyme in Walker cells

The enzyme that catalyses the aerobic reduction of CB 1954 to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide has been isolated from Walker cells [40]. By comparison of partial protein sequences, coenzymes, substrate and inhibitor specificities, and spectroscopic data, the enzyme has now been identified as a form of DT diaphorase (NAD(P)H dehydrogenase (quinone), EC 1.6.99.2). This name arose because of its then unique ability to use either

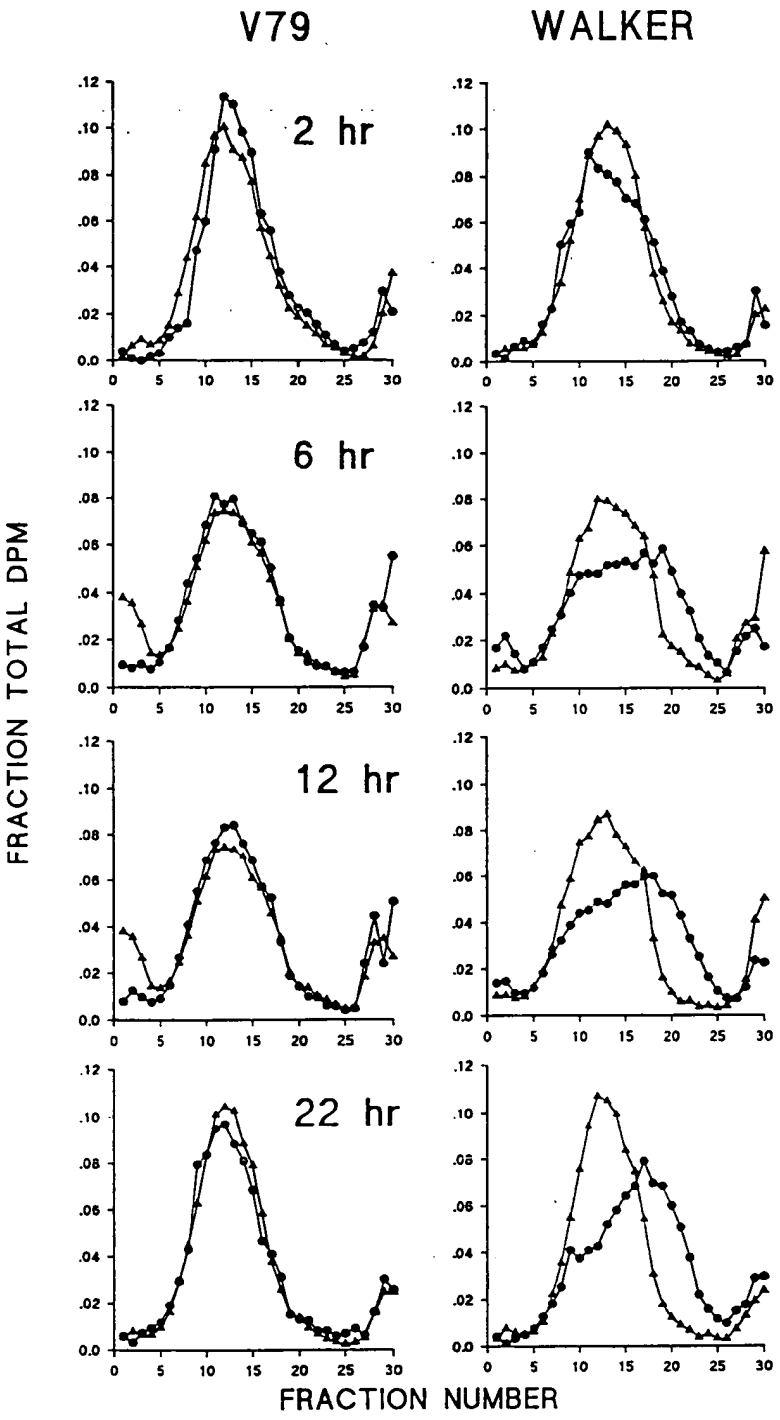

Fig. 2. The formation of DNA interstrand crosslinks in Walker cell DNA, but not V79 cell DNA, during a continuous exposure to 1 μM CB 1954. Control (▲) untreated ^{14}C-labeled and CB 1954-treated (●) ^{3}H-labeled cells were co-analyzed by alkaline sucrose gradient sedimentation. The direction of sedimentation is from left to right. DNA from CB 1954-treated Walker cells sediments further into the gradient than does that from untreated control cells or treated V79 cells. This indicates the presence of higher molecular weight DNA characteristic of the formation of DNA-DNA interstrand crosslinks [39]. Data from [30].

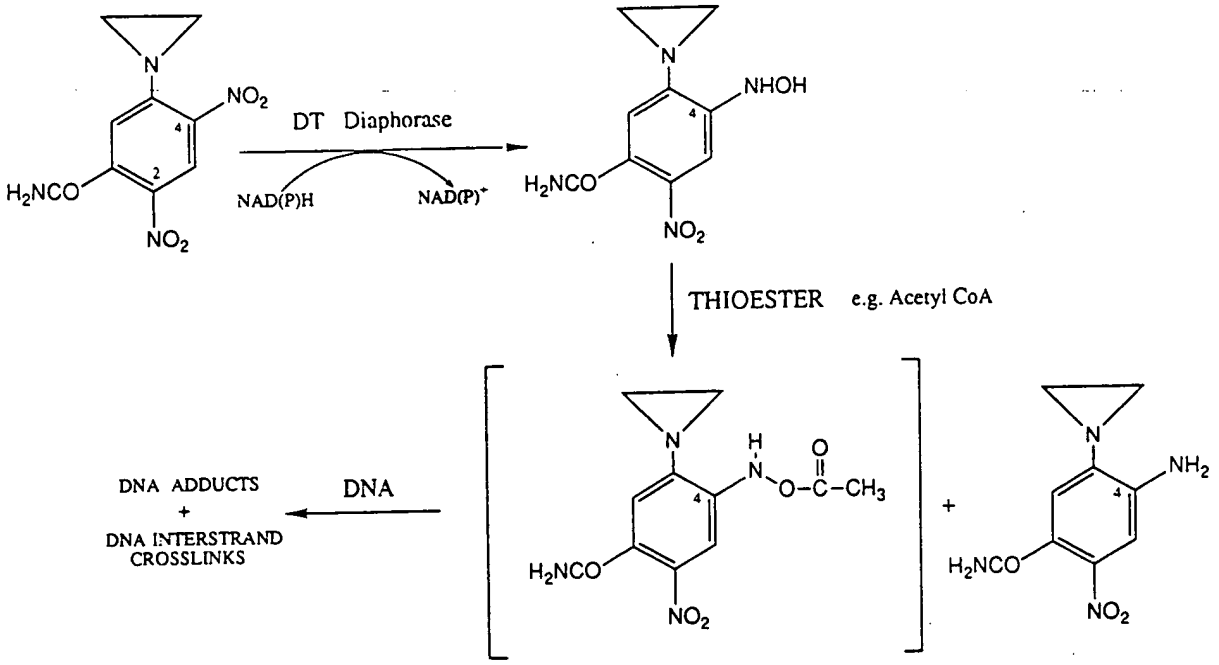

Fig. 3. The bioactivation of CB 1954. The initial step is the reduction of CB 1954 by the enzyme DT diaphorase to form 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. This hydroxylamine derivative can react with thioesters to produce DNA reactive species. It is postulated that this is the N-acetoxy derivative. The major product of this reaction is, however, 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide that does not react readily with DNA. Formation of 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide is in competition with the production of DNA binding products.

NADH or NADPH as co-factors in its reduction of quinone substrates. At the time of its discovery these two co-factors were abbreviated as DPNH and TPNH, respectively. As well as its ability to use both of these co-factors, this enzyme is also remarkable in that it can simultaneously transfer two electrons to its substrate. The primary cellular role of this enzyme appears to be the detoxification of quinones by directly catalyzing a two-electron reduction of these compounds to form the hydroquinone. This avoids the cytotoxicity resulting from the redox cycling produced by a one-electron reduction of quinones and the resulting generation of superoxide radicals (for a review see [41]). As well as detoxifying quinones, DT diaphorase is involved in the metabolism of a number of chemotherapeutic agents (reviewed by [42]). Presumably the mechanism by which DT diaphorase can directly transfer 2 electrons to a quinone is used in the 4-electron reduction of CB 1954. However, CB 1954 is intrinsically reduced 1.6×10^4 times slower than the quinone compound, menadione, and transfer of reducing equivalents from the enzyme to CB 1954 is slow and very rate limiting [43]. This relatively slow rate of reduction may be required to avoid saturation of the second activation step discussed above. To emphasize the point that DT diaphorase can alone account for a cell's sensitivity to CB 1954; the addition of the enzyme to the culture medium of V79 cells produced a dramatic increase in their sensitivity towards CB 1954 [31].

It would be predicted from these results that inhibition of DT diaphorase would protect Walker cells from the cytotoxic effects of CB 1954 by inhibiting the formation of the active species 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. Dicoumarol is a diagnostic inhibitor of DT diaphorase. Interesingly, 2-phenyl-AICA and AICA, two compounds that had been previously been reported to be antagonists of the anti-tumour effects of CB 1954 were also shown to be an inhibitor of DT diaphorase. Caffeine was also shown to be a novel inhibitor of this enzyme [44]. These inhibitors produced a dose-dependent reduction in cell death, but

only when they were present at the same time as CB 1954 [44] (Fig. 5). This is consistent with this protective action being due to the inhibition of the activating enzyme as opposed to other mechanisms. Inhibitors also markedly reduced the amount of crosslinked DNA in the CB 1954 co-treated cells; relative to that in those cells treated with CB 1954 alone [44]. Emphasizing that these compounds were indeed acting by inhibiting DT diaphorase in the cell, the same three compounds potentiated the cytotoxicity of menadione; a quinone compound that will be detoxified by this enzyme [44] (Fig. 5).

In summary, it is the reduction of CB 1954 by DT diaphorase that defines a cell's sensitivity to this agent.

3.C The lack of sensitivity of human cells towards CB 1954

The identification and characterization of the enzyme that reduces CB 1954 to the toxic crosslinking derivative (5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide) as DT diaphorase, renewed the possibility of identifying human tumour types with a sensitivity similar to the Walker carcinoma. This was because this enzyme is known to be widely distributed in mammalian tissues (see [41]) and its activity is considerably elevated in chemically-induced preneoplastic nodules in rat liver when compared with normal tissue [45]. The enzyme is also selectively induced in the livers of tumour-bearing animals [46, 47]. In addition, DT diaphorase activity is present in a variety of tissues and cell lines of human origin. High activity has been detected in stomach and abdominal adipose tissue [48, 49] and elevated activity has been reported in human tumour cell lines of breast, brain and liver origin [50, 51]. There is a marked increase in the activity of DT diaphorase in human colonic carcinomas when compared with the enzymatic activity of the surrounding normal colonic mucosa [52]. No significant differences between the Walker, rat liver and human forms of this enzyme have been reported. Both the rat and human forms of DT diaphorase have been cloned and sequenced and human DT diaphorase cDNA and proteins are 83% and 85% homologous with the rat liver cytosolic cDNA and protein, respectively [53]. Both are inducible cytosolic flavoproteins encoded by a single gene. The human protein is biochemically very similar to the rat protein, only small differences between Km values for the substrates menadione and NADH having been found [48]. Thus it might be predicted that human or rat DT diaphorase would metabolize CB 1954 in a manner similar to the protein from Walker cells and that the cytotoxicity resulting from the bioactivation of CB 1954 might be observed in human tumours expressing significant levels of this enzyme.

A number of human cell lines were shown to contain DT diaphorase levels comparable to those found in Walker and some other rat cell lines [33] (Fig. 6). The rat cell lines were all sensitive to CB

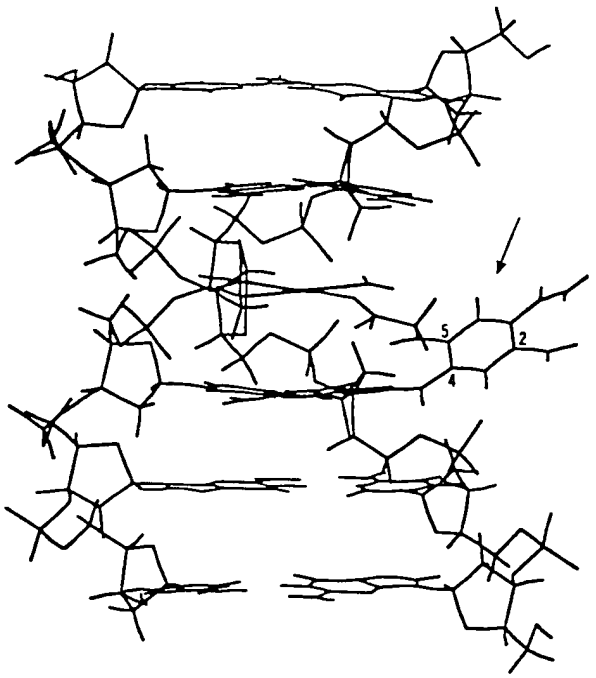

Fig. 4. The proposed structure of the interstrand crosslink produced by CB 1954. The 4-hydroxylamine, after activation reacts predominantly with the C8 position of deoxyguanosine. Modelling studies indicate that the aziridine function can then react on the O6 position of a deoxyguanosine on the opposite strand of DNA to produce the observed crosslinks. Such a DNA interstrand crosslink would be unique. This could explain why CB 1954 produces a very high crosslink frequency and why, in cells, these crosslinks are very persistent and not removed readily by DNA repair processes.

1954 and the resulting cell kill approached that obtained in Walker cells. Thus Walker cells are not uniquely sensitive towards CB 1954. Human cell lines were, on the other hand, all dramatically less toxically effected by CB 1954 with between a 500 and 5000 higher dose of the agent being required to produce a comparable cytotoxic response to that obtained in cells of rat origin [33] (Fig. 7). In contrast to the large difference in their cytotoxic response towards CB 1954, both the rat and human cell lines were similarly affected by the 4-hydroxylamino derivative of CB 1954 [33] (Fig. 7). The fact that human cells were sensitive to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide suggested that their resistance towards CB 1954 was not due to any failure to activate further the hydroxylamine nor to an intrinsic resistance to the DNA adducts formed. It was therefore suggested that CB 1954 was reduced differently by the human form of DT diaphorase as compared to the rat form [33].

In order to investigate this proposal, the human form of DT diaphorase was purified to homogeneity from Hep G2 cells [33]. Although DT diaphorase has been extensively studied, the most common source of the enzyme is rat liver and information regarding the human protein was limited. However, as might be predicted from the large degree of homology between the rat and human forms of DT diaphorase, the biochemical properties of the Hep G2 and Walker forms of the enzyme, with respect to cofactors and the reduction of menadione, were very similar [33]. In contrast, significant differences were observed in the ability of DT diaphorase isolated from either Hep G2 or Walker cells in their ability to reduce CB 1954 to the active 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide derivative. Although, both forms of the enzyme produced the 4-hydroxylamino derivative as the single product, the human Hep G2 form of the enzyme was intrinsically less able to carry out this reduction (Fig. 8) and the K_{cat} value is over six-fold higher for the Walker cell form of the enzyme ($4.1\,min^{-1}$) than for the human DT diaphorase ($0.64\,min^{-1}$) [33]. Interestingly, CB 1954 inhibits the reduction of menadione by DT diaphorase and is a competitive inhibitor (with respect to NADH) of the Hep G2 enzyme. The ki value (130μM) indicates that CB 1954 can bind reason-

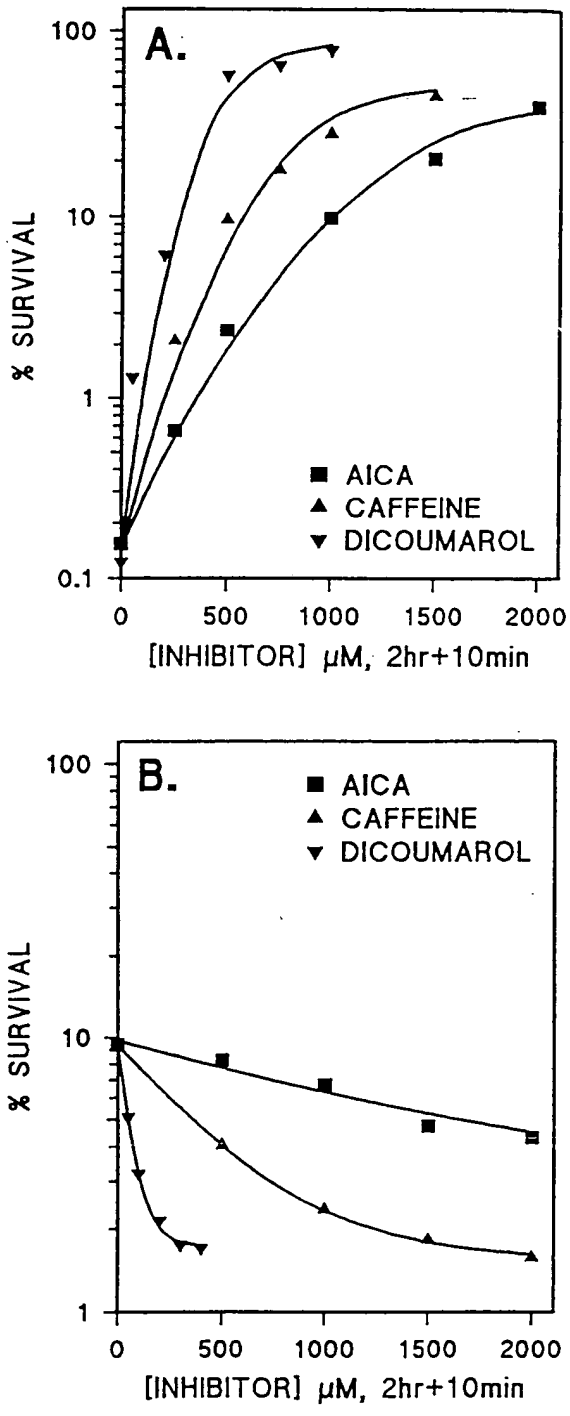

Fig. 5. The effects of inhibitors of DT diaphorase on the survival of Walker cells treated with either (A) 100nM CB 1954 or (B) 20μM menadione. Cells were treated for 2hr at 37°C in the presence of various concentrations of the inhibitors as shown and then assayed for colony forming ability. Inhibitors were added 10min prior to the drug. Inhibition of DT diaphorase protects against CB 1954 cytotoxicity but potentiates menadione cytotoxicity. Data from [44].

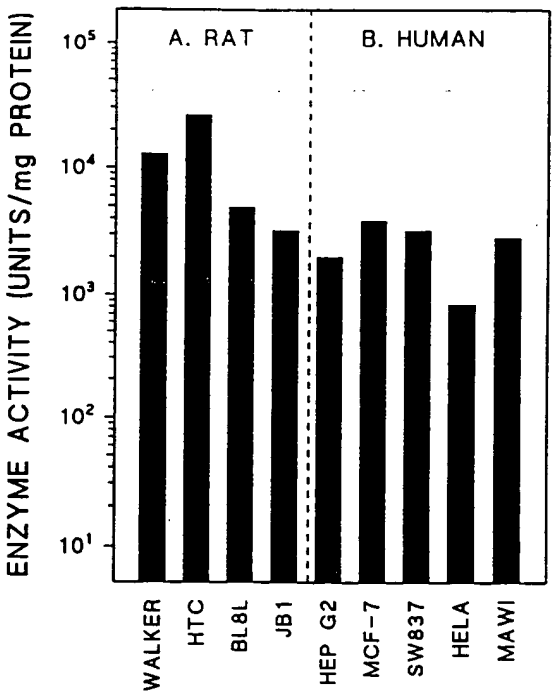

Fig. 6. The activity of the enzyme DT diaphorase in cell lines of either (A) rat or (B) human origin. DT diaphorase activity was assayed employing menadione (10μM) as substrate and cytochrome C (70μM) as a terminal electron acceptor. Activity was defined as the cytochrome C reduction inhibited by 1μM dicoumarol and expressed as nmoles cytochrome C reduced per minute (units) per mg of total protein at 37°C. Data from [33].

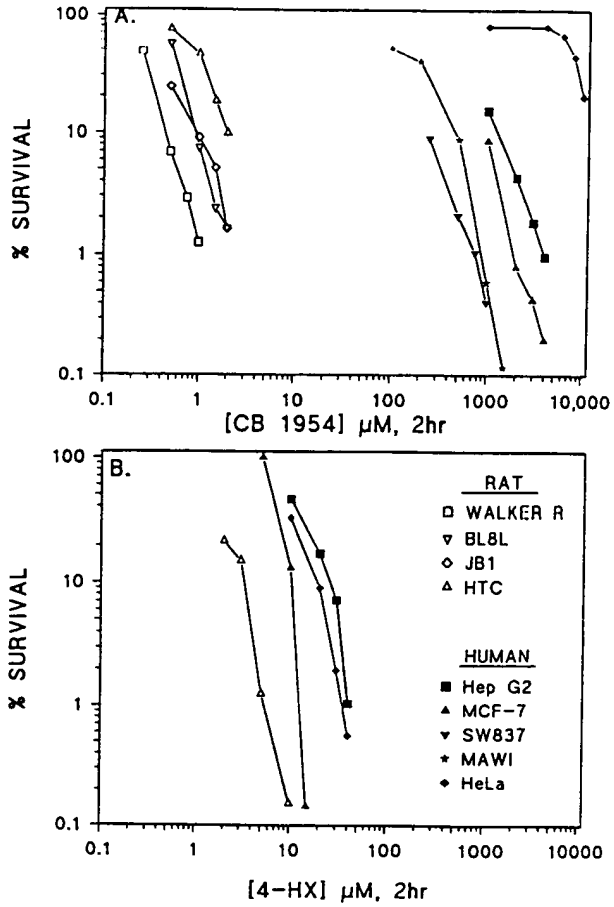

Fig. 7. The effect of (A) CB 1954 or (B) 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide on the survival of cells of either rat or human origin. Cells were exposed to the agent for 2hr at 37°C and then assayed for colony forming ability. Drug concentrations are plotted on a logarithmic scale. All the human cell lines are resistant to CB 1954. They are, however, sensitive to its active, 4-hydroxylamine, form. Data from [33].

ably well to the enzyme (much better than might be inferred from the km of the enzyme for CB 1954). Thus CB 1954 can be considered an inhibitor of, rather than a substrate for, human DT diaphorase [33].

The intrinsic inability of human DT diaphorase to produce the required cytotoxic species from CB 1954 accounts for the lack of sensitivity of human cells towards this agent.

4. CB 1954 – The future

4.A Analogues of CB 1954

Although CB 1954 has been shown to be poorly reduced to the 4-hydroxylamine product by human DT diaphorase it is possible that an analogue of CB 1954 could be found which would be a better substrate for the human enzyme. Such a compound could be able to exploit the raised levels of this enzyme in human tumours, which have been reported to exist (see above). Now that the mechanism of action of CB 1954 is fully understood, the criteria for an analogue would be a) a reducible nitro group ortho to the aziridine, b) the product of its reduction must the the 4-electron hydroxylamino derivative and this must be able to react with thioesters and c) low cytotoxicity in cells with low levels of DT diaphorase. Utilizing analogues synthesized during the original development a number of interesting compounds have been examined which illustrate this approach.

Substitution of the carboxamide by a chloro group at the C1 position (CB 10-207) resulted in a 6.58 fold increase in the rate of reduction by human DT diaphorase when compared to CB 1954 [54]. This compound was previously shown to have no effect on the growth of the Walker tumour *in vivo* although it was quite toxic to the whole animal (LD_{50} = 60 mg/ml). This improved rate of reduction and toxicity may have reflected in the sensitivities of a range of human cell lines towards this compound when compared with CB 1954. However, cells deficient in DT diaphorase were also cytotoxically effected by this compound suggesting that it may be activated by other enzymes apart from DT diaphorase [54].

Substitution of the carboxamide by an isobutylcarboxylate moiety (CB 10-200) results in a compound that is reduced 40-times faster by human DT diaphorase than CB 1954 itself [55]. CB 10-200 (5-(aziridin-1-yl)-2,4-dinitro-1-isobutylbenzoate) is cytotoxic in human cell lines with raised levels of DT diaphorase but not in cell lines with low levels of the enzyme [55]. These findings indicate that CB 10-200 retains the high selectivity found with CB 1954 whilst being more readily reduced to its active form by the human form of DT diaphorase. However, CB 10-200 is not as cytotoxic towards human cell lines as CB 1954 is towards rat cell lines with comparable DT diaphorase levels.

4.B Potentiation of CB 1954 cytotoxicity by reduced pyridine nucleotides

The toxicity of CB 1954 towards human cells was greatly enhanced by the presence of NADH (when foetal calf serum was present in the culture medium) [56] (Fig. 9). The actual molecule that causes this effect is nicotinamide mononucleoside (reduced) (NRH) [56]. NRH is a cofactor for rat DT diaphorase [43] and is generated from NADH by enzymes in foetal calf serum [57]. NRH can enter the cell and stimulate the activity of human DT diaphorase towards CB 1954, although the precise mechanism of this stimulation is not known [56].

The intrinsic differences in the two forms of the enzyme means that, even in the presence of NADH

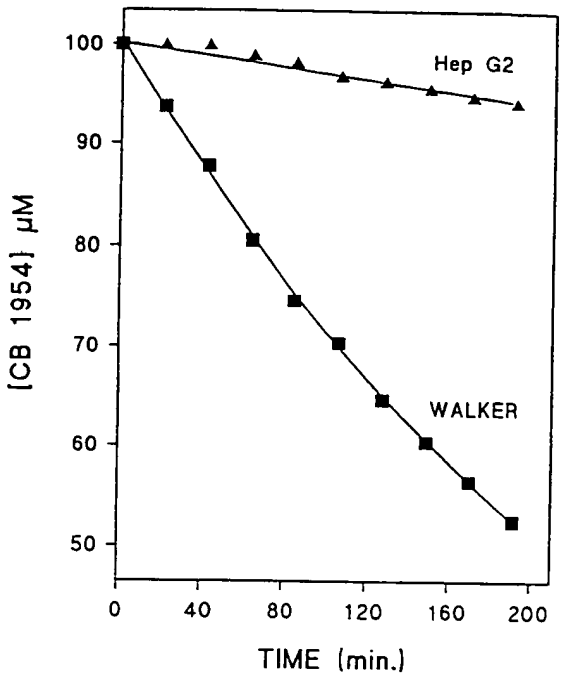

Fig. 8. The rate of reduction of CB 1954 by 6×10^7 units/ml of either the Walker (rat) or Hep G2 (human) forms of DT diaphorase. Reduction was monitored by HPLC and NADH was used as a cofactor at an initial concentration of 500μM. CB 1954 is reduced much slower by the human enzyme than by the rat form of DT diaphorase. The product of the reduction is 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide in both cases. Data from [33].

(or NRH), the cytotoxicity of CB 1954 in human cells is still much less than in rat cells of equivalent DT diaphorase levels. However, the technique should also work with CB 1954 analogues and may increase the chances of a selective antitumour agent being developed.

4.C Antibody directed enzyme prodrug therapy (ADEPT)

A recent innovation in the attempt to produce a tumour-selective cytotoxic chemotherapy has been the Antibody Directed Enzyme Prodrug Therapy (ADEPT) approach [58, 59]. A tumour-selective monoclonal antibody (or fragment) is conjugated to an enzyme that is capable of bioactivating a prodrug. Therapy proceeds along a multistage line. A conjugate of an enzyme with a monoclonal anti-

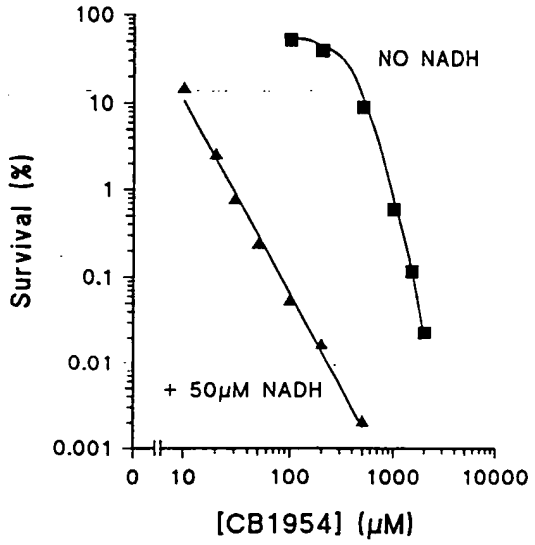

Fig. 9. The potentiation of CB 1954 cytotoxicity by NADH. Human MAWI cells were incubated in the presence or absence of 50μM NADH plus the doses of CB 1954 indicated, for 2hr at 37°C and then assayed for colony forming ability. NADH substantially potentiates CB 1954 cytotoxicity. The mechanism involves the metabolism of NADH to nicotinamide mononucleoside (reduced). This compound is still a cofactor for DT diaphorase and can stimulate the activity of human DT diaphorase towards CB 1954. The effect is much less marked in human cell lines with low DT diaphorase levels. Data from [56].

body is allowed to localize at the site of the tumour. The unlocalized conjugate is either given time to be eliminated from the body, or is hastened on its way by a 'clearance' antibody. A non-toxic prodrug is then administered and activated at the site of the tumour by the bound conjugate [60]. The enzymes carboxy-peptidase G2 [61], alkaline phosphatase [62], β-lactamase [63, 64], penicillin amidase [65], and cytosine deaminase [66] have been considered. In these cases the activating chemical event of the prodrug is hydrolysis.

Reductive processes are also capable of activating prodrugs. The increase in cytotoxicity accompanying bioreduction of the 4-nitro group of CB 1954 is up to a 100,000 fold on a dose basis. So large an increase in cytotoxicity makes CB 1954 an attractive ADEPT candidate prodrug.

In ADEPT endogenous enzymes are not being exploited and, indeed, it is fundamental to the concept that the prodrug (such as CB 1954) is not activated by normal human enzymes. The biochemical differences between enzymes from different mammalian species would not be expected to be that great. Despite being a mammalian enzyme, rat DT diaphorase could be suitable in this respect, because the human form of DT diaphorase is much less able to metabolize CB 1954 than is the rat form. Nitroreduction of CB 1954 by DT diaphorase may therefore be a possible ADEPT system. Although there is an exploitable difference between the human and rat forms of DT diaphorase, candidate enzymes for ADEPT have tended to be bacterial in origin. Nitroreductase proficient strains of bacteria are sensitive towards CB 1954 [32] and a nitroreductase enzyme has been purified from *E. coli B* [67]. This enzyme, like DT diaphorase, is also capable of reducing CB 1954 in air to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. In contrast to DT diaphorase, which can only reduce the 4-nitro group of CB 1954, the *E. coli* nitroreductase can reduce either (but not both) nitro groups of CB 1954 to the corresponding hydroxylamino species (Fig. 10). The two hydroxylamino species are formed in equal proportions and at the same rates [34] (Fig. 11). However, no products are formed in which both nitro groups have been reduced. Thus, once one nitro group has been reduced, the *E. coli* nitroreductase can not then reduce the other nitro group. 5-(Aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide is a potent cytotoxic agent capable of producing DNA-DNA interstrand crosslinks in cells [31]. In contrast the 2-hydroxylamino species is less cytotoxic and can not produce interstrand crosslinks. It is, however, much more cytotoxic than CB 1954 [31]. Importantly, CB 1954 is reduced about 90-fold more rapidly by the *E. coli* nitroreductase than by DT diaphorase [67]. In the presence of V79 cells (normally insensitive to CB 1954) the *E. coli* nitroreductase is capable of activating CB 1954 extracellularly to a cytotoxic form [34]. As well as being able to reduce CB 1954, the *E. coli* nitroreductase shares some other biochemical properties with DT diaphorase. It can reduce menadione [67] and utilizes either NADH or NADPH as a cofactor [67]. However, it is a much smaller protein (24kD) [67] than DT diaphorase (33.5kD) [40] and there is no obvious sequence homology between the two enzymes [67]. Conjugates between DT diaphorase or the *E.*

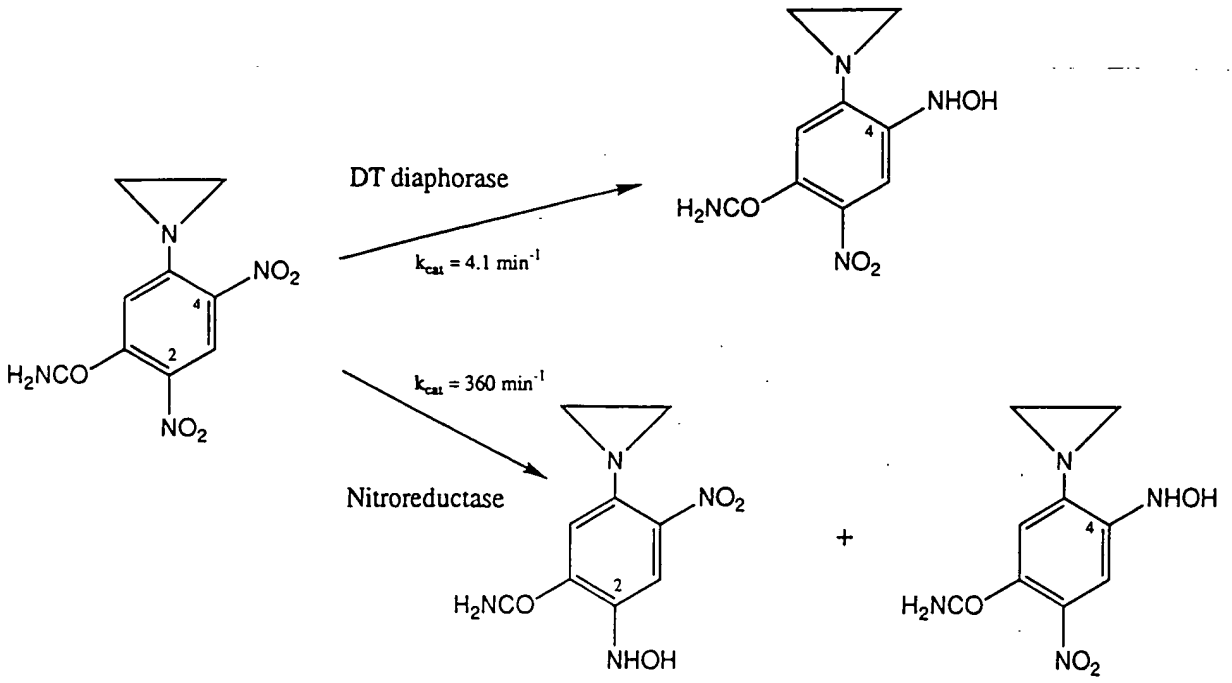

Fig. 10. The products formed by the reduction of CB 1954 by DT diaphorase or *E. coli* nitroreductase. DT diaphorase only forms 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide but the nitroreductase generates both the 2- and 4-hydroxylamino forms in equal proportions. The 2-hydroxylamine is less cytotoxic than the 4-hydroxylamine but is is still much more cytotoxic than CB 1954 itself. CB 1954 is intrinsically reduced about 90-fold faster by the nitroreductase than by Walker DT diaphorase.

coli nitroreductase have been made with the tumour selective antibody A5B7. The conjugates retained both enzyme and antibody activity (unpublished data).

For a nitroreductase enzyme to be used in ADEPT a cofactor would also have to be present to supply a source of reducing equivalents. The biogenic cofactors for reductases, NADH and NADPH are unsuitable in this respect being rapidly oxidized and degraded by serum enzymes [57]. However, NAD^+ is more stable and can be reduced to NADH by the serum enzyme, L(+)lactate dehydrogenase (E.C. 1.1.1.27) [57] and NADH generated from NAD^+ by serum can drive the bioreductive activation of CB 1954 [57]. It is possible that this innate ability of serum to reduce NAD^+ can be practically exploited in a reductive ADEPT system.

A novel approach to this problem has been in the development of enzyme selective cofactors. This development started with the observation that very simple reduced pyridinium compounds were still good cofactors for DT diaphorase [43]. However, they are not substrates for the serum enzymes that metabolise NAD(P)H and are therefore serum stable. Similarly the *E. coli* nitroreductase can use some of these compounds and can even use some that are not cofactors for DT diaphorase. Such a compound is nicotinate riboside (reduced) (unpublished data). An added advantage of this cofactor is that it does not potentiate CB 1954 cytotoxicity in human cells (presumably because it is not a cofactor for DT diaphorase) [43] and should make this prodrug more selective in ADEPT.

These observations suggest that the pharmacological limitations of NADH need not be an obstacle to the use of bioreductive enzymes for ADEPT therapy. Given its high activity for CB 1954 the *E. coli* nitroreductase is a very attractive enzyme for ADEPT.

5. Concluding remarks

The selective bioactivation of CB 1954 by DT

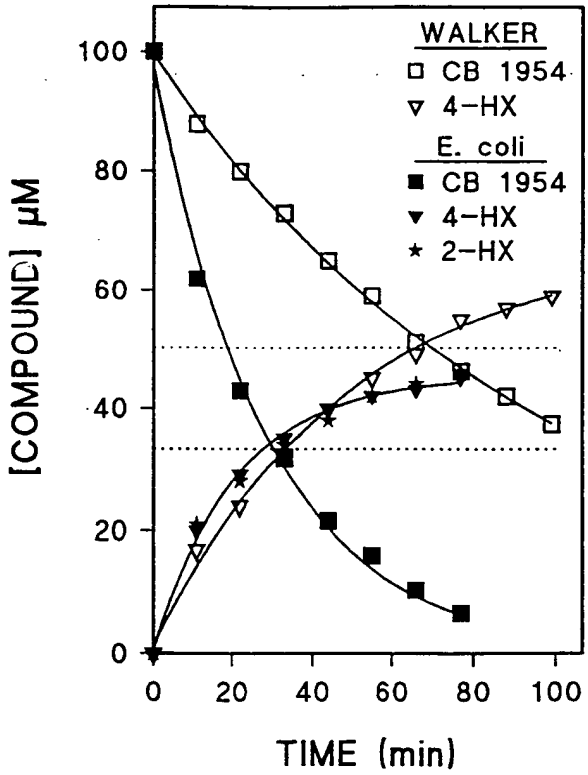

Fig. 11. The aerobic reduction of CB 1954 and the formation of products by either 2μg/mL *E. coli* nitroreductase or 35μG/mL Walker DT diaphorase. The *E. coli* nitroreductase reduces CB 1954 much more rapidly than Walker DT diaphorase but forms a mixture of the 2- and 4-hydroxylamines. In contrast DT diaphorase only forms 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (4-HX). Data from [34].

diaphorase offered the potential of new anti-cancer strategies. This compound possesses the latent cytotoxicity required of a prodrug and antibodies raised against the enzyme could establish from individual biopsies those tumours that may have been responsive to prodrug therapy. Unfortunately this approach is thwarted by the intrinsic poor rate of reduction of CB 1954 by the human form of DT diaphorase. However, the development of analogues that are more readily reduced by the human enzyme and therefore more cytotoxic makes it very conceivable that a derivative will be found with the required rate of bioactivation and specifity, but with the minimum of toxic side effects. This type of compound may still have a place in the chemotherapy of human tumours. An alternate role for CB 1954 is as a prodrug in ADEPT therapy. In combination with the conjugated nitroreductase enzyme, CB 1954 itself has an important role to play in this innovative approach to cancer chemotherapy. Perhaps, at last, CB 1954 has found its 'human tumour to treat'.

Acknowledgements

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